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<b>(21) International Application Number:</b> PCT/US92/09038 <b>(22) International Filing Date:</b> 23 October 1992 (23.10.92) <b>(30) Priority data:</b> 07/781,898      24 October 1991 (24.10.91)      US <b>(71) Applicants:</b> CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 55 Shattuck Street, Boston, MA 02115 (US). MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US). <b>(72) Inventors:</b> ATALA, Anthony ; 38 Pine Street, Weston, MA 02193 (US). VACANTI, Joseph, P. ; 6 Hillcrest Parkway, Winchester, MA 01890 (US). FREEMAN, Michael, R. ; 68 W. Rutland Square, Boston, MA 02116 (US). LANGER, Robert, S. ; 77 Lombard Street, Newton, MA 02158 (US).		<b>(74) Agents:</b> ENGELLENTER, Thomas, J. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US). <b>(81) Designated States:</b> AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> NEOMORPHOGENESIS OF UROLOGICAL STRUCTURES IN VIVO FROM CELL CULTURE <b>(57) Abstract</b> <p>Methods and artificial matrices for the growth and implantation of urological structures and surfaces are disclosed in which urothelial cells are grown in culture on biodegradable, biocompatible, fibrous matrices formed of polymers, such as polyglycolic acid, polylactic acid, or other polymers which degrade over time. The cells can be cultured <i>in vitro</i> until an adequate cell volume and density has developed for the cells to survive and proliferate <i>in vivo</i>. Alternatively, when adequate cell numbers for implantation are available, the cells can be attached to the matrix and implanted directly, without proliferation <i>in vivo</i>. The implants approximate the desired urological structure to be replaced or repaired, such as the kidney, ureter, bladder, urethra, and the like. Implantation is followed by remodeling through cell growth and proliferation <i>in vivo</i>. In another aspect of the invention, techniques are disclosed for selectively extracting or harvesting urothelial cells either from excised urological tissue <i>in vitro</i> or from intact urological tissue <i>in vivo</i> by treating the tissue with a digestive enzyme, such as collagenase.</p>		

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NEOMORPHOGENESIS OF UROLOGICAL STRUCTURES IN VIVO  
FROM CELL CULTURE

5           The United States Government has rights in  
this invention pursuant Grant No. 6M 26698 awarded by  
the National Institutes of Health.

Background of the Invention

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          The technical field of this invention is  
prosthetic surgery and cell culturing. In  
particular, the invention pertains to methods and  
implantable urological structures formed on  
15 biocompatible artificial matrices for the  
reconstruction or repair of urological structures.

          Shortage of native urothelium places  
significant constraints on the success of surgical  
20 reconstruction in a wide variety of urologic  
conditions. Investigators have recognized this  
problem and have looked for alternatives for  
urothelial replacement. A variety of natural  
tissues, such as omentum and seromuscular grafts, and  
25 synthetic materials, such as polyvinyl sponge and  
Teflon, have been tried in experimental and clinical  
settings.

          Unfortunately, these attempts have not  
30 produced satisfactory structural and functional  
replacement. Synthetic materials alone cannot  
replace selective transport functions of mucosal  
tissue and commonly are lithogenic in the urinary  
tract.

35

Bowel tissue has been used for urinary tract reconstruction for many years, however it is also associated with numerous complications. These include metabolic abnormalities, such as hyperchloremic metabolic acidosis arising from electrolyte reabsorption, infection, perforation, stone formation, increased mucous production, diverticular formation, and malignancy. Despite these problems, incorporation of intestinal segments into the urinary system has increased because of the lack of a suitable alternative.

A homologous substitute for urothelial tissues would be ideal. Recent work involving hypospadias reconstruction using bladder mucosal grafts demonstrates the potential of autologous replacement. See, for example, Hendren and Reda, Vol. 21 J. Pediatric Surgery, pp. 181-192 (1986) and Ransley et al., Vol. 58 Brit. J. Urology, pp. 331-333 (1986). However, the limited availability of urothelial tissue for patients requiring major reconstruction involving the kidney, ureter, bladder or urethra generally precludes its use.

The use of reconstructed sheets of autologous urothelium in urinary tract reconstruction would be ideal for a variety of surgical procedures and would have the added advantage of avoiding immunologic rejection. Autologous skin keratinocytes have been used successfully in the treatment of

extensive burn wounds. See, for example, Green et al., Vol. 76 Proc. Nat'l. Acad. Sci. pp. 5665-5668 (1979) and Burke et al., Vol. 194 Ann. Surgery, pp. 413-428 (1981). Keratinocytes derived from the urethral meatus have also been used in urethral grafts for hypospadias repair in humans as described in Romagnoli et al, Vol. 323 New England J. medicine, pp. 527-530 (1990).

- 10 U.S. Serial No. 679,177 entitled "Chimeric Neomorphogenesis Of Organs By Controlled Cellular Implantation Using Artificial Matrices" filed March 26, 1991, and U.S. Serial No. 933,018 entitled "Chimeric Neomorphogenesis Of Organs Using Artificial  
15 Matrices" filed November 20, 1986, by Joseph P. Vacanti and Robert S. Langer, herein incorporated by reference, describe methods and means whereby cells having a desired function are grown on polymer scaffolding using cell culture techniques, followed  
20 by transfer of the cell polymer scaffold into a patient at a site appropriate for attachment, growth and function after attachment and equilibration to produce a functional organ equivalent. Success depends on the ability of the implanted cells to  
25 attach to the surrounding environment and to stimulate angiogenesis. Nutrients and growth factors are supplied during cell culture allowing for attachment, survival or growth as needed.

After the structure is implanted and growth and vascularization take place, the resulting organoid is a chimera formed of parenchymal elements of the donated tissue and vascular and matrix elements of the host. The polymer scaffolding used for the initial cell culture is constructed of a material which degrades over time and is, therefore, not present in the chimeric organ. Vascular ingrowth following implantation allows for normal feedback mechanisms controlling the soluble products of the implanted cells. The preferred material for forming the matrix or support structure is a biodegradable, artificial polymer, for example, polyglycolic acid, polyorthoester or polyanhydride, which is degraded by hydrolysis at a controlled rate and reabsorbed.

These materials provide the maximum control of degradability, manageability, size and configuration. In some embodiments, these materials are overlaid with a second material, such as gelatin or agarose, to enhance cell attachment. The polymer matrix must be configured to provide both adequate sites for attachment and adequate diffusion of nutrients from the cell culture to maintain cell viability and growth until the matrix is implanted and vascularization has occurred. The preferred structure for organ construction is a branched, fibrous, tree-like structure formed of polymer fibers having a high surface area which results in a relatively shallow concentration gradient of nutrients, wastes and gases so as to produce uniform cell growth and proliferation.

U.S. Serial No. 933,018 and U. S. Serial No. 679,177 disclose several examples of the successful culturing and implantation of hepatocytes, intestine, and pancreas cells, with subsequent normal function, including production and secretion of bioactive molecules. Examples of such molecules include growth hormone from pituitary cells, insulin and glycogen from pancreatic cells and clotting factors from liver cells. As described in these applications, however, there is a need for a different type of functioning "organ," one which provides primarily a structural function. Examples of types of cells which are useful in these applications include cartilage, bone and muscle cells.

15

It is an object of the present invention to provide a method and means for designing, constructing and utilizing artificial matrices as temporary scaffolding for cellular growth and implantation of urological structures.

It is a further object of the invention to provide biodegradable, non-toxic matrices which can be utilized for cell growth, both in vitro and in vivo, as supports for urological structures.

It is a still further object of the invention to provide biodegradable, non-toxic matrices which can be utilized for cell growth both in vitro and in vivo, to replace or to repair urological structures.

It is another object of this invention to provide an in vitro system in which cells will retain their normal morphology and cell function.

Summary of the Invention

Methods and artificial matrices for the growth and implantation of urological structures and 5 surfaces are disclosed.

In one aspect of the invention, urothelial cells are grown in culture on biodegradable, biocompatible, fibrous matrices formed of polymers, 10 such as polyglycolic acid, polylactic acid, or other polymers which degrade over time, as a function of hydrolysis of the polymer backbone into innocuous monomers. The matrices are designed to allow adequate nutrient and gas exchange to the cells until 15 engraftment and vascularization at the site of engraftment occurs. The cells can be cultured in vitro until an adequate cell volume and density has developed for the cells to survive and proliferate in vivo, or maintained in vitro for the purpose of 20 manufacturing bioactive molecules. Alternatively, when adequate cell numbers for implantation are available, the cells can be attached to the matrix and implanted directly, without proliferation in vitro. One advantage of the matrices is that they 25 can be cast or molded such that the cells, when seeded on them, approximate the desired urological structure to be replaced or repaired, such as the ureter, bladder, uretra, and the like. Implantation is followed by remodeling through cell growth and 30 proliferation in vivo.



In another aspect of the invention, techniques are disclosed for establishing cultures of transitional uroepithelial cells which can be expanded in vitro using explant culture techniques, 5 or in vivo using graft techniques. For example, as a first step in this approach, urothelial cells can be disassociated into single cell preparations, placed onto a synthetic substrate, and returned directly to animals or humans, where the normal reparative 10 signals will direct regeneration, repair and remodelling. In addition, this approach allows the exploitation of the in vivo environment for the enhancement of growth in concert with the preservation of differentiated function.

15

In yet another aspect of the invention, techniques are disclosed for selectively extracting or harvesting urothelial cells either from excised urological tissue in vitro or from intact urological 20 tissue in vivo by treating the tissue with a digestive enzyme, such as collagenase.

Detailed Description

U.S. Serial No. 679,177 entitled Chimeric Neomorphogenesis Of Organs By Controlled Cellular Implantation Using Artificial Matrices filed March 26, 1991, by Joseph P. Vacanti and Robert S. Langer describes a technique of placing dispersed cell types onto synthetic, biodegradable polymer fibers in vitro which have been configured to produce high cell densities by allowing adequate diffusion of nutrients and waste as well as gas exchange. This technique has been applied to urothelial cells for the purpose of creating implants of urological structures, such as the urethers, bladder, urethra and the like.

In the preferred method, polymer fibers are placed in culture media containing urothelial cells, where the urothelial cells attach to the fibers in multiple layers and retain their normal configuration, which appears to be essential for the urothelial cells to maintain their normal function and remain viable. This technique also allows transplantation of the polymer cell scaffold into animals without disrupting the complex of attached urothelial cells. Transplantation of this complex containing a high density of normally-functioning urothelial cells with a large surface area into an animal allows the cells to obtain adequate nutrition by diffusion and successful engraftment of functioning urothelial tissue, even in the initial absence of vascularization.

The examples below demonstrate that it is possible to grow in culture on fibers of biodegradable polymers urothelial cells that appear to be morphologically and functionally normal and will proliferate to a cell density sufficient to allow implantation of the cell polymer scaffold in animals and successful engraftment with formation of a new tissue equivalent as the polymer resorbs. The examples also demonstrate that the polymer fiber scaffold is essential in that neither injection of free urothelial cells nor implantation of the polymer fibers without attached urothelial cells results in urological tissue formation.

15           The method and matrices providing structural and functional urological equivalents using bioabsorbable, artificial substrates as temporary scaffolding for cellular transfer and implantation reaffirms the principles first outlined in U.S. Serial No. 933,018 filed November 20, 1986:

1.   Every structure in living organisms is in a dynamic state of equilibrium, undergoing constant renewal, remodeling and replacement of functional tissue which varies from organ to organ and structure to structure.

2.   Dissociated structural cells tend to reform structure, depending on the environment in which they are placed and the degree of alteration which they have undergone.

3. Cell shape is determined by cytoskeletal components and attachment to matrix plays an important role in cell division and differentiated function. If dissociated cells are placed into mature tissue as a suspension without cell attachment, they may have difficulty finding attachment sites, achieving polarity and functioning because they begin without intrinsic organization. This limits the total number of implanted cells which can remain viable to organize, proliferate, and function.

The latter principle is a key point in the configuration of the urothelial cell support matrices. For an organ to be constructed in tissue culture and subsequently successfully implanted, the matrices must have sufficient surface area and exposure to nutrients such that cellular growth and differentiation can occur prior to the ingrowth of blood vessels following implantation. After implantation, the configuration must allow for diffusion of nutrients and waste products and for continued blood vessel ingrowth as cell proliferation occurs.

25

Urothelial cells are initially isolated and cultured using a novel technique. Digestive enzymes such as collagenase, trypsin or EDTA can be used to isolate urothelial cells for recovery and seeding purposes. In one approach, a urothelial biopsy sample can be taken and the mucosa stripped away and placed in an enzyme solution, such as an aqueous collagenase solution having an enzyme concentration from about 0.1 to about 0.75 percent. The urothelial

cells will be liberated and can then be collected by centrifuging or the like. Alternatively, a catheter can be introduced into the bladder to fill the bladder with an enzyme solution (e.g., a milder  
5 collagenase solution from about 0.05 to about 0.40 percent collagenase). Following irrigation of the bladder and collection of the rinses, urethelial cells can again be collected and used to seed a polymeric matrix.

10

Once the cells have begun to grow and cover the matrix, they are implanted in a patient at a site appropriate for attachment, growth and function. One of the advantages of a biodegradable polymeric matrix  
15 is that angiogenic and other bioactive compounds can be incorporated directly into the matrix so that they are slowly released as the matrix degrades in vivo. As the cell polymer structure is vascularized and the structure degrades, the cells will differentiate  
20 according to their inherent characteristics.

In the preferred embodiment, the matrix is formed of a bioabsorbable or biodegradable, synthetic polymer, such as a polyanhydride, polyorthoester or  
25 polyglycolic acid. In some embodiments, attachment of the cells to the polymer is enhanced by coating the polymers with compounds, such as basement membrane components, agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin,  
30 glycosaminoglycans, mixtures thereof, and other materials having properties similar to biological matrix molecules known to those skilled in the art of cell culture. All polymers must meet the mechanical and biochemical parameters necessary to provide

adequate support for the cells with subsequent growth and proliferation. Factors, including nutrients, growth factors, inducers of differentiation or dedifferentiation, products of secretion, immunomodulators, inhibitors of inflammation, regression factors, biologically-active compounds which enhance or allow ingrowth of the lymphatic network or nerve fibers, and drugs, can be incorporated into the matrix or provided in conjunction with the matrix. Similarly, polymers containing peptides, such as the attachment peptide RGD (Arg-Gly-Asp) can be synthesized for use in forming matrices.

15 A presently-preferred polymer is Polyglactin, developed as absorbable synthetic suture material, a 90:10 copolymer of glycolide and lactide, manufactured as Vicryl® braided absorbable suture (Ethicon Co., Somerville, New Jersey) See, Craig et al.: A Biological Comparison Of Polyglactin 910 And Polyglycolic Acid Synthetic Absorbable Sutures. Vol. 141, Surg., p. 1010 (1975). Polyglycolide fibers can be used as supplied by the manufacturer. Other shapes can be fabricated using one of the following methods:

Solvent Casting. A solution of polymer in an appropriate solvent, such as methylene chloride, is cast as a branching pattern relief structure. After solvent evaporation, a thin film is obtained.

Compression Molding. Polymer is pressed (30,000 psi) into an appropriate pattern.

Filament Drawing. Filaments are drawn from the molten polymer.

Meshing. A mesh is formed by compressing 5 fibers into a felt-like material.

The polymers can be characterized with respect to mechanical properties, such as tensile strength using an Instron tester, for polymer molecular weight by gel permeation chromatography (GPC), glass transition temperature by differential scanning calorimetry (DSC) and bond structure by infrared (IR) spectroscopy; with respect to toxicology by initial screening tests involving Ames assays and in vitro teratogenicity assays and implantation studies in animals for immunogenicity, inflammation, release and degradation studies. In vitro cell attachment and viability can be assessed using scanning electron microscopy, histology and quantitative assessment with radioisotopes.

At the present time, a mesh-like structure formed of fibers, which may be round, scalloped, flattened, star shaped, solitary or entwined with other fibers is preferred. The use of branching fibers is based upon the same principles which nature has used to solve the problem of increasing surface area proportionate to volume increases. All multicellular organisms utilize this repeating

branching structure. Branching systems represent communication networks between organs, as well as the functional units of individual organs. Seeding and implanting this configuration with cells allows  
5 implantation of large numbers of cells, each of which is exposed to the environment of the host, providing for free exchange of nutrients and waste while neovascularization is achieved.

10           The polymeric matrix may be made flexible or rigid, depending on the desired final form, structure and function. For reconstruction of kidney structures such as the renal pelvis as well as for reconstruction of bladder, sheet-like matrices may be  
15 preferable in many instances. For reconstruction of the urethra and/or ureters, tubular matrices will often be preferable.

          Cells may be derived from the host, a  
20 related donor or from established cell lines. In one variation of the method using a single matrix for attachment of one or more cell lines, the scaffolding is constructed, such that initial cell attachment and growth occur separately within the matrix for each  
25 population, for example, urothelial and non-urothelial cell populations. Alternatively, a unitary scaffolding may be formed of different materials to optimize attachment of various types of cells at specific locations. Attachment is a  
30 function of both the type of cell and matrix composition.



The following non-limiting examples demonstrate actual attachment of cell preparations to bioerodible, artificial polymers in cell culture and implantation and engraftment of this polymer-cell scaffold into animals. These samples describe methods of harvesting primary uroepithelial cells from rabbits and demonstrate the use of biodegradable polyglycolic acid polymer matrices as vehicles for delivery and maintenance of urothelium in host animals to create new urothelial tissue in vitro.

EXAMPLES

- Young adult New Zealand white rabbits (Charles River Labs, Wilmington, MA) were used as cell donors for all experiments. Young adult athymic nu/nu mice (Charles River Labs) were used as cell recipients. The animals were housed individually, allowed access to food and water ad lib, and maintained at 12 hr light and dark intervals.
- 10 Anesthesia for rabbits was carried out with an intramuscular injection of ketamine and rompum. Mice were anesthetized with Enflurane by cone administration.
- 15 Biodegradable polymers were obtained as nonwoven meshes of polyglycolic acid, a synthetic polymer of hydroxyacetic acid (Davis and Geck, Danbury, CT). The average fiber diameter was 15 Mm. Interfiber distances varied between 0 and 200 Mm.
- 20 Dimensions of the polymer meshes used for implantation were 0.75 x 0.75 cm. Polymers were sterilized in ethylene oxide and sealed in aluminum foil until implantation.
- 25 Rabbit urothelial cells (RUC) for transplantation were isolated using the following method of cell harvesting. After the induction of anesthesia, the shaved abdomens of rabbits were painted with betadine and opened using sterile
- 30 technique. The bladders were isolated, the ureters ligated and the urethras cannulated. The bladders were rinsed with 3-5 ml of sterile phosphate buffered saline (PBS) and filled with a buffered collagenase solution comprised of 0.1-0.75% collagenase B from
- 35 Cl. histolyticum (Boehringer Mannheim, Indianapolis, IN) and sealed.

The ligated bladders were then placed in a 50ml conical tube with the remaining volume filled with PBS and incubated in a water bath at 37°C. Incubation time was varied. Urothelial cell suspensions were recovered from the donor bladders, washed with a 4:1 mixture of DMEM and Ham's F12 medium (Gibco, Grant Island, NY) supplemented with 25 mg/ml adenine, 0.244 mg/ml biotin, 0.0136 ng/ml triiodothyronine, 5 mg/ml insulin, 5 mg/ml transferrin, and penicillin and streptomycin, and pelleted in a clinical centrifuge. Cell resuspension and centrifugation was carried out an additional two times.

The cells were then counted with a hemacytometer and their viability determined by the Trypan Blue exclusion method. The urothelial cells were then seeded onto polyglycolic acid biodegradable polymers and were maintained at 37°C in a humidified atmosphere of 95%air/5% CO<sub>2</sub>. After 1 to 4 days in culture, the polymers were implanted into the retroperitoneum, subcutaneous tissue or omentum of athymic mice. Implants were then retrieved at sacrifice at 5, 10, 20 and 30 days. Polymers implanted without cells served as controls. Implants were examined histologically and by western blot.

Implants recovered from host animals were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin.

Protein fractions enriched in cytoskeletal proteins, according to protein isolation protocol described in Freeman and Sueoka, Vol. 84, PNAS, pp. 5808-5812 (1987), were separated on 10% polyacrylamide gels according to the procedure of Laemmli. Approximately 15 mg protein was loaded per lane. Proteins were electrophoretically blotted onto Immobilon-P blotting membranes (Millipore, Bedford, MA). Non-specific sites were blocked in: 1% (w/v) Carnation non-fat dry milk, 0.5% (v/v) Tween 20, 150 mM NaCl, 20 mM Tris base. AE1 and AE3 monoclonal antibody mixture (Boehringer Mannheim) was incubated at 2 mg/ml in blocking buffer for 2 hr at room temperature.

15

After repeated washes with blocking buffer, the membrane was incubated for 2 hr with anti-mouse IgG linked to alkaline phosphatase (Bio-Rad, Richmond, CA) at 1:3000 dilution. Membranes were washed repeatedly in blocking buffer, then in substrate buffer (100M Tris base, 100mM NaCl, 50 mM MgCl<sub>2</sub>, pH9.5) Colorimetric detection was with the NBT/BCIP color reagent (Bio-Rad) until the bands were adequately visualized.

25

The objective of these experiments was to determine whether newly harvested urothelial cells would attach to biodegradable polymers in vitro and whether these polymers would serve as a suitable synthetic substrate for delivery and maintenance of epithelial architecture in vivo. Explant techniques have generally been used for the establishment of primary cultures of uroepithelial cells. However, for the experiments described here, the use of

explant cultures would not provide an adequate number of cells. Therefore, a method was developed that would allow isolation of bladder cell populations efficiently, and in which large quantities of cells enriched in uroepithelia can be obtained rapidly and quantitatively. One approach was to excise freshly-irrigated bladders from rodents and to treat the mucosal surface with collagenase by incubating the proteolytic enzyme solution within the enclosed, ligated bladder for various times.

Initial attempts at cell harvest with this method using Fisher rats resulted in the recovery of less than  $5 \times 10^4$  viable cells per bladder and, although a limited sequence of implants into synergetic hosts was carried out using these cells, this approach was later abandoned. In subsequent trials, in which bladders were obtained from New Zealand white rabbits, adequate numbers of cells were obtained reproducibly. Attempts to optimize the cell harvest method by varying incubation time and collagenase concentration resulted in establishment of one preferred condition of incubation for 2 hr at  $37^\circ\text{C}$  in 0.5% collagenase. Assessment of cell viability using the Trypan Blue exclusion method indicated that these conditions resulted consistently in the recovery of the greatest number of viable cells. Quantitation of cell recovery by cell counting over an extended period indicated that this method resulted in an average recovery of  $1.7 \times 10^6 \pm 1.01 \times 10^5$  viable cells/bladder (n=32).

Morphological analysis of these cell populations plated onto plastic surfaces by phase contrast microscopy indicated that they were composed largely of uroepithelial cells, with very little fibroblast contamination evident within the first five days in culture. With maintenance of the cultures for later times, fibroblast outgrowth occurred. This result suggests that this method of cell harvest results in recovery of populations enriched in urothelium and that these populations can be maintained as relatively homogeneous cultures at least for several days.

Rabbit uroepithelial cells (RUC) obtained using this cell harvest method were seeded directly onto nonwoven polyglycolic polymers of approximately 0.56 cm<sup>2</sup> surface area in culture and allowed to attach from between 1 to 4 days. Cell attachment to the polymers occurred readily as assessed by phase contrast microscopy with only limited numbers of floating cells, indicating that under our conditions, cultures enriched in healthy, viable cells were obtained routinely. In most experiments polymer-cell allografts were implanted into host animals after incubation in culture medium overnight.

Allografts containing RUC were surgically implanted in the mesentery, retroperitoneum, or subcutaneous tissue of athymic (nude) mice (not greater than three implants per animal) and recovered at varying times after implantation. Twenty-eight adult New Zealand white rabbits were used as donors

for cell harvest. Thirty-three nude mice were used as recipients. Not including host animals lost by attrition, a total of 58 cell-polymer scaffolds were recovered from host animals at 5 to 30 day time points. A total of 17 polymers implanted without cells and maintained in vivo for the respective times served as controls.

Histologic analysis demonstrated an inflammatory response, which exhibited both an acute phase and a chronic foreign body reaction. Fibroblast infiltration and proliferation were seen up to 10 days after implantation. Vascular ingrowth was apparent in most implants by 5 days. Control polymers elicited similar inflammatory and angiogenic responses, suggesting that these responses were largely due to the polymers.

Epithelial cells were evident in a substantial fraction of RUC-polymer implants recovered at each time point, including the allografts maintained in vivo for 30 days. Epithelial layers were frequently found associated with polymer fibers. The number of implants containing identifiable urothelial cells was highest at the 5 day time point (89%). Single cell layers were evident in 10 day implants and multiple epithelial cell layers were evident in 20 day and 30 day implants. Recognizable epithelial populations were not evident in any of the 17 control polymers.

These results indicate that RUC can survive and can remain associated with the polymer fibers at the ectopic implant sites even after extended periods in vivo.

Western blot analysis of protein fractures obtained from 30 day RUC-polymer implants demonstrated the presence of a prominently-expressed 40kDa cytokeratin present in rabbit bladder uroepithelium, identified using the AE1 and AE3 anticytokeratin monoclonal antibodies. In this assay, this cytokeratin expression pattern can be distinguished from that seen using protein from mouse skin. Cytokeratins were not identifiable in protein fractions from control polymers implanted without cells. This result suggests the potential for maintenance of uroepithelial differentiation in implants maintained in host animals for long periods at ectopic sites. This result also confirms the histologic assessment that eosinophilic cell types present in the polymer allografts at late time points are RUC.

In summary, the feasibility of using biodegradable polymers as delivery vehicles for urothelial cell populations in vivo has been demonstrated. Primary rabbit urothelial cells (RUC) were found to attach readily to unwoven polyglycolic acid polymers in vitro. Polymer-RUC allografts were found to contain viable RUC after implantation times for as long as 30 days at ectopic sites in athymic mice, although some RUC cell death in the implants was noted. Polymers were seen to evoke an angiogenic response, which should allow RUC populations to survive, and possibly proliferate, after extended periods in vivo. RUC oriented themselves spatially along the polymer surfaces and RUC populations appeared to expand from one to several cell layers in thickness with extended times of implantation.



Western blot analysis confirmed the presence of a cytokeratin protein associated with rabbit bladder urothelium in implants recovered after 30 days. This results suggests that some cell  
5 type-specific functions associated with bladder differentiation may be retained in implants after extended periods. We have also presented an efficient method of uroepithelial cell harvest which will allow the efficacy of reconstitution of  
10 urothelial cell growth, structure and function to be studied more extensively in this model system.

Reconstructive procedures involving the bladder, ureters, urethra and kidney frequently  
15 employ bowel segments, despite the potential for numerous complications. The benefit of using bladder mucosa in these procedures is self-evident, however a suitable urothelial substitute for bowel would require the creation of autologous urothelial sheets,  
20 most likely originating from limited biopsy material. Harvesting and expansion of autologous urothelium, in the manner successfully employed with skin, may not be possible, due to the relatively limited proliferative potential of differentiated  
25 uroepithelium in vitro. This limitation may be overcome by use of molecular regulators of uroepithelial growth and differentiation.

The present study provides evidence that the creation of urothelial structures de novo from diassociated urothelial cells can be achieved in vivo using biodegradable polymers as delivery vehicles.

5 Unwoven polyglycolic acid polymers provided an adequate surface for urothelial cell attachment and were found to serve as suitable substrates for urothelial survival, and possibly growth, in vivo. Further, RUC on these polymers were found to orient

10 themselves spatially in the host animals, and to exhibit some aspects of urothelial cell differentiation (cytokeratin expression), even after extended periods. Significantly, contiguous layers of presumptive uroepithelium were identified in most

15 of the RUC-polymer implants found to contain eosinophilic cells, even though the cell harvest procedure involved the isolation and seeding of suspensions of single cells. This indicates that these polymers can support the spontaneous

20 reorientation of uroepithelium into layered structures resembling normal transitional cell architecture.

Some differentiated cell types, such as

25 chondrocytes and hepatocytes, have been found to remain functionally differentiated and in some cases to expand in vivo on nonwoven polyglycolic acid or polylactic acid polymers. The polymer fibers provide sites for cell attachment, the reticular nature of

30 the polymer lattice allows for gas exchange to occur over considerably less than limiting distances, and the polymers evoke host cell responses, such as angiogenesis which promote cell growth.

Synthetic polymers can also be modified in vitro before use, and can carry growth factors and other physiologic agents such as peptide and steroid hormones, which promote proliferation and differentiation. The malleability of the synthetic polymer used in this study also should allow for the creation of cell-polymer implants manipulated into preformed configurations (e.g., tubes in the case of urothelium).

10

In genitourinary reconstruction, cell-polymer allografts can be implanted along existing urinary structures in manipulated forms resembling normal in vivo architecture. The polyglycolic acid polymer undergoes biodegradation over a four month period; therefore as a cell delivery vehicle it permits the gross form of the tissue structure to be reconstituted in vitro before implantation with subsequent replacement of the polymer by an expanding population of engrafted cells.

The newly-developed urinary structure can thereby mimic the gross configuration of the polymer scaffold, be composed of transplanted uroepithelial cells and their derivatives, be functional in situ, and possibly be translatable to another site in a reconstructive procedure.

Although this invention has been described with reference to specific embodiments, variations and modifications of the method and means for constructing urothelial implants by culturing 5 urothelial cells on matrices having maximized surface area and exposure to the surrounding nutrient-containing environment will be apparent to those skilled in the art. Such modifications and variations are intended to come within the scope of 10 the appended claims.

We claim:

1. A system for growing a prosthetic urological structure comprising:

a biocompatible, biodegradable polymeric matrix in a nutrient environment; and  
5 urothelial cells attached the matrix, wherein the matrix is structured to provide free exchange of nutrients and waste to attached cells in the absence of vascularization.

10 2. The system of claim 1 wherein the polymer is selected from the group consisting of polyanhydride, polyorthoester, polyglycolic acid, polylactic acid and combinations thereof.

15 3. The system of claim 1 further comprising coatings on the polymer selected from the group consisting of basement membrane components, agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin, glycosaminoglycans and mixtures  
20 thereof.

4. The system of claim 1 wherein the matrix forms a flexible structure conformable to biological surface.

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5. The system of claim 1 wherein the matrix is formed of fibers.

6. A method for making a chimeric  
30 urological structure comprising:

providing a biocompatible, biodegradable polymeric matrix in a nutrient environment and

attaching urothelial cells to the  
35 matrix to form a hybrid structure.

7. The method of claim 6 wherein the polymer is selected from the group consisting of polyanhydride, polyorthoester and polyglycolic acid.

5 8. The method of claim 6 further comprising coating the polymer with a material selected from the group consisting of basement membrane components, agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin, 10 glycosaminoglycans, attachment peptides, functional equivalents, and mixtures thereof.

9. The method of claim 6 wherein the matrix is formed as a flexible structure conformable 15 to a biological surface.

10. The method of claim 6 wherein the nutrient environment is in vivo further comprising attaching the cells on a matrix and implanting the 20 attached cells on the matrix in an animal without first proliferating the cells on the matrix in vitro.

11. The method of claim 6 further comprising attaching the cells on a matrix, 25 proliferating the cells on the matrix in vitro in a nutrient media, then implanting the cells on the matrix in vivo.

12. The method of claim 6 further 30 comprising implanting the structure to form a urological prosthesis.

13. A method isolating urothelial cells for culturing purposes, the method comprising:

exposing urological tissue to an enzyme solution; and

5 collecting urothelial cells which are dissociated by said solution.

14. The method of claim 13 wherein the step of exposing said tissue to an enzyme solution further  
10 comprises exposing said tissue to a collagenase solution.

15. The method of claim 13 wherein the concentration of the solution ranges from about 0.05  
15 percent to about 0.75 percent collagenase.

16. The method of claim 13 wherein the step of exposing said tissue further comprises excising a tissue biopsy, isolating the mucosa from the biopsy  
20 and placing the mucosa in said solution.

17. The method of claim 13 wherein the step of exposing said tissue further comprises irrigating the interior of a bladder in vivo with said solution.  
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18. The method of claim 13 wherein the step of collecting urothelial cells further comprises centrifuging a solution containing the dissociated cells.

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19. The method of claim 13 wherein the method further comprises:

providing a biocompatible,  
biodegradable polymeric matrix in a nutrient  
5 environment and

attaching urothelial cells to the  
matrix to form a hybrid structure.

20. The method of claim 19 wherein the  
10 method further comprises culturing the cells and  
matrix together to create a urological structure and  
then implanting the structure to form a urological  
prosthesis.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/09038

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61L27/00; C12N5/00; A61F2/06		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	A61L ; C12N ; A61F	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,9 012 604 (J. VACANTI) 1 November 1990  see page 16, line 26 - page 27, line 2; claims see page 10, line 30 - page 16, line 2 ---	1-9, 13-15, 18-19
Y	WO,A,8 803 785 (J. VACANTI) 2 June 1988  see page 10 - page 17; claims & US PATENT APPLICATION 933018 (20-11-1986) cited in the application ---  -/--	1-9, 13-15, 18-19
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28 JANUARY 1993	19. 02. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	G.COUSINS- VAN STEEN	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
Y	THE JOURNAL OF UROLOGY vol. 133, no. 5, May 1985, pages 866 - 869 TACHIBANA E.A. 'URETERAL REPLACEMENT USING COLLAGEN SPONGE TUBE GRAFTS' see the whole document	1-9, 13-15, 18-19
A	US,A,4 645 669 (L. REID) 24 February 1987	
P,X	THE JOURNAL OF UROLOGY vol. 148, no. 2PT2, August 1992, pages 658 - 662 ATALA E.A. 'FORMATION OF UROTHELIAL STRUCTURES IN VIVO FROM DISSOCIATED CELLS ATTACHED TO BIODEGRADABLE POLYMER SCAFFOLDS IN VITRO'	1-9, 13-15, 18-19

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9209038  
SA 66082

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

28/01/93

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US-A-4645669	24-02-87	None	

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